A Herpes Simplex Virus Type 1 Human Asymptomatic CD8+ T-Cell Epitopes-Based Vaccine Protects Against Ocular Herpes in a Humanized HLA Transgenic Rabbit Model

Permalink
https://escholarship.org/uc/item/0n03b4hs

Journal
Investigative Ophthalmology & Visual Science, 56(6)

ISSN
1552-5783

Authors
Srivastava, Ruchi
Khan, Arif A
Huang, Jiawei
et al.

Publication Date
2015-06-19

DOI
10.1167/iovs.15-17074

License
CC BY 4.0

Peer reviewed
A Herpes Simplex Virus Type 1 Human Asymptomatic CD8⁺ T-Cell Epitopes-Based Vaccine Protects Against Ocular Herpes in a “Humanized” HLA Transgenic Rabbit Model

Ruchi Srivastava, Arif A. Khan, Jiawei Huang, Anthony B. Nesburn, Steven L. Wechsler, and Lbachir BenMohamed

1Laboratory of Cellular and Molecular Immunology, Gavin Herbert Eye Institute, University of California Irvine, School of Medicine, Irvine, California, United States
2Department of Microbiology and Molecular Genetics, University of California Irvine, School of Medicine, Irvine, California, United States
3Center for Virus Research, University of California Irvine, Irvine, California, United States
4Department of Molecular Biology & Biochemistry
5Institute for Immunology, University of California Irvine, School of Medicine, Irvine, California, United States

With a staggering one billion individuals worldwide currently carrying herpes simplex virus type 1 (HSV-1), herpes remains one of the most prevalent viral infections of the eye. Ocular herpes infection causes a spectrum of clinical manifestations ranging from blepharitis, conjunctivitis, and dendritic keratitis to disciform stromal edema and blinding stromal keratitis (HSK). In the United States alone, over 450,000 people have a history of recurrent ocular HSV infection and disease still is lacking. In the present study, preclinical vaccine trials of nine asymmetric (ASYMP) peptides, selected from HSV-1 glycoproteins B (gB), and tegument proteins VP11/12 and VP13/14, were performed in the “humanized” HLA-transgenic rabbit (HLA-Tg rabbit) model of ocular herpes. We recently reported that these peptides are highly recognized by CD8⁺ T cells from “naturally” protected HSV-1-seropositive healthy ASYMP individuals (who have never had clinical herpes disease).

**RESULTS.** All mixtures elicited strong and polyfunctional IFN-γ- and TNF-α-producing CD107⁺ CD8⁺ cytotoxic T cells, associated with a significant reduction in death, ocular herpes infection, and disease ($P < 0.015$).

**CONCLUSIONS.** The results of this preclinical trial support the screening strategy used to select the HSV-1 ASYMP CD8⁺ T-cell epitopes, emphasize their valuable immunogenic and protective efficacy against ocular herpes, and provide a prototype vaccine formulation that may be highly efficacious for preventing ocular herpes in humans.

Keywords: HSV-1, HLA transgenic rabbit, animal model, T cell, glycoprotein B, VP11/12, VP13/14, vaccine, ocular herpes
and/or gD), which deliver protective epitopes, nonprotective epitopes, and maybe even pathogenic epitopes (i.e., infection- or disease-enhancing epitopes, reviewed previously). Thus, although these traditional vaccines were intended to target only HSV-specific protective B- and T-cell immunity, antigen processing also might have generated HSV-derived T-cell epitopes that elicit nonprotective T-cell responses and possibly even harmful T-cell responses. We recently found that HSV-1 seropositive asymptomatic (ASYMP) patients (with a history of numerous episodes of recurrent ocular herpes disease) tend to develop CD4+ T cells and CD8+ T cells1,2,4-6 that strongly recognize a subset of HSV-1 epitopes that differs from HSV-1 epitopes that are strongly recognized by CD4+ and CD8+ T cells from HSV-1 seropositive healthy asymptomatic (ASYMP) individuals (who have never had clinical herpes disease), and vice versa. The former epitopes are designated as “asymptomatic” or SYMP epitopes and the later are designated “asymptomatic” or ASYMP epitopes.

In the present study, based on the above observation in humans, we tested the hypotheses that: (1) an ocular herpes vaccine that exclusively includes ASYMP CD8+ T-cell epitopes (while excluding SYMP T-cell epitopes) will be highly immunogenic and protective in the “humanized” HLA-A*02:01 transgenic rabbit (HLA-Tg rabbit) model of ocular herpes3,7,18 and (2) immunization of HLA-Tg rabbits with a mixture of ASYMP human herpes T-cell epitopes selected from several HSV-1 Ags will generate multiepitopes and polyfunctional protective CD8+ T-cell responses. To test these hypotheses, and to pave the way toward moving ASYMP multiepitopic peptide vaccines to clinical application, we used our recently developed HLA-Tg rabbit model. This preclinical animal model of ocular herpes mounts “humanized” HLA-restricted CD8+ T-cell responses to human herpes.

We report here that immunization of HLA-Tg rabbits with mixtures of human HSV-1 ASYMP human epitopes, selected from gB and tegument proteins VP11/12 and VP13/14, induced potent and polyfunctional HSV-specific IFN-γ- and TNF-α-producing CD107+CD8+ cytotoxic T cells. These CD8+ T-cell responses were positively associated with a significant reduction in death, ocular herpes infection, and corneal herpetic disease (P < 0.015). The findings of this preclinical study strongly suggest that mixtures of HSV-1 ASYMP epitopes display promising immunogenic and protective properties to be considered in the next generation of ocular herpes vaccines, and confirm the relevance of “humanized” HLA-Tg rabbits as a useful preclinical animal model for assessing the protective efficacy of human CD8+ T-cell epitope-based vaccines against ocular herpes.

### Materials and Methods

**HLA-A*02:01 Tg Rabbits**

A colony of HLA Tg rabbits maintained at University of California, Irvine (Irvine, CA, USA) were used for all experiments. The HLA-Tg rabbits were derived from New Zealand White rabbits. The HLA-Tg rabbits retain their endogenous rabbit MHC locus and express human HLA-A*02:01 under the control of its normal promoter. Before this study, the expression of HLA-A*02:01 molecules on the peripheral blood mononuclear cells (PBMC) of each HLA-Tg rabbit was confirmed by FACS analysis. Briefly, rabbit PBMCs were stained with 2 μL of anti–HLA-A2 monoclonal antibodies (mAb; clone BB7.2; BD-Pharmering, San Jose, CA, USA), at 4°C for 30 minutes. The cells were washed and analyzed by flow cytometer using a LSRII (Becton Dickinson, Mountain View, CA, USA). The acquired data were analyzed with FlowJo software (TreeStar; Ashland, OR, USA). All rabbits used in these studies had a similar high level of HLA-A*02:01 expression (>90%). This eliminated any potential bias due to variability of HLA-A*0201 molecul levels in different animals. New Zealand White rabbits (non-Tg control rabbits), purchased from Western Oregon Rabbit Co. (Philomath, OR, USA), were used as controls. All rabbits were housed and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic Research, the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; Frederick, MD, USA), and National Institutes of Health (NIH; Bethesda, MD, USA) guidelines.

**Virus Production**

Strain McKrae HSV-1 was used in this study. The virus was triple plaque purified and prepared as described previously.20-22

**Peptide Vaccines**

We selected 9 potential peptide epitopes from HSV-1: three from gB (gB17,25, gB32,350, and gB61,650), three from VP11/12 (VP11/1266-74, VP11/12220-228, VP11/12202-210), and three from VP13/14 (VP13/14286-294, VP13/14501-512, VP13/14544-552; Table 1). Peptide epitopes were synthesized by 21st Century Biochemicals (Marlboro, MA, USA). All peptides were HPLC purified to a purity of 95% to 98%.

**Immunization**

Rabbits (HLA-Tg, 8–10 weeks) with similar, high expression of HLA-A*02:01 molecules (>90%) were used, as described above. Groups of age-matched HLA-A*02:01 rabbits (n = 10 each) were immunized subcutaneously twice (2 weeks apart) with a mixture of three CD8+ peptide epitopes (each at 100 μM) delivered with the CD4+ T helper epitope (PADRE) emulsified in CpG (ODN 2007) in a total volume of 200 μL. As a negative control, a group of HLA-Tg rabbits (n = 10) were injected with adjuvant alone. Two weeks after the final immunization, both eyes were ocularly infected (challenges) as described above.

**Clinical Scores**

Rabbits were examined for ocular disease and survival for 30 days after challenge. Ocular disease was determined by a masked investigator using fluorescein staining and slit-lamp examination before challenge, and on days 1, 4, 7, 10, 14, and 21 thereafter. A standard 0 to 4 scale: 0, no disease; 1, 25%; 2, 50%; 3, 75%; and 4, 100% staining, was used.

**Quantification of Infectious Virus**

Tears were collected from both eyes using a Dacron swab (type 1; Spectrum Laboratories, Los Angeles, CA, USA) on days 3, 5, and 7 after challenge. Individual swabs were transferred to a 2 mL sterile cryogenic vial containing 1 mL culture medium and stored at −80°C until use. The HSV-1 titers in tear samples were determined by standard plaque assays on RS cells as described previously.25

**PBMC Isolation**

Blood (20 mL) was drawn from each rabbit into a yellow-top Vacutainer Tube (Becton Dickinson). Sera were isolated by centrifugation for 10 minutes at 800g. We isolated PBMCs by gradient centrifugation using leukocyte separation medium.
Flow Cytometry Analysis

We analyzed PBMC by flow cytometry. The following antibodies were used: mouse anti-rabbit CD8 (clone MCA1576F; AbD Serotec, Oxford, UK), mouse anti-rabbit CD4 (clone MCA799F, AbD Serotec), mouse anti-human CD107a, CD107b, and rat anti-mouse IFN-γ (clone XMG1.2), and mouse anti-human TNF-α, TNF-β (BD-Pharmingen). For surface staining, mAbs were added to the cells and incubated for 45 minutes on ice and in the dark. Cells were washed again with Perm/Wash and FACS buffer, and fixed in PBS containing 2% paraformaldehyde (Life Technologies, Rockville, MD, USA). Aliquots of freshly isolated PBMC also were cryopreserved in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen for future testing.

Statistical Analyses

Data for each assay were compared by ANOVA and Student’s t-test using GraphPad Prism version 5 (La Jolla, CA, USA). Differences between the groups were identified by ANOVA and multiple comparison procedures, as we described previously. Data are expressed as the mean ± SD. Results were considered statistically significant at P < 0.05.

RESULTS

Selection of Asymptomatic HLA-A*02:01-Restricted T-Cell Epitopes From HSV-1 gB, VP11/12, and VP13/14 Proteins

Nine ASYMP peptide epitopes that induced strong CD8+ T-cell responses from “naturally” protected HSV-1-seropositive healthy ASYMP individuals (who have never had clinical herpes disease), but not from SYMP individuals, were selected from HSV-1 gB, VP11/12, and VP13/14 proteins. We recently reported that these peptide epitopes induced significantly higher expression of granzyme B (GzmB), granzyme K (GzmK), perforin (PFN), degranulation marker CD107a/b, and IFN-γ by CD8+ T cells from ASYMP, compared to SYMP individuals.5,4,25,26 Briefly, in 10 sequentially studied HLA-A*0201 positive, HSV-1 seropositive ASYMP individuals, the most frequent, robust, and polyfunctional gB-specific CD8+ T-cell responses, as assessed by a combination of tetramer, IFN-γ, ELISPOT, CFSE proliferation, CD107a/b cytotoxic degranulation, expression of GzmB, GzmK, PFN, were directed mainly against gB17-25, gB342-350, and gB561-569 epitopes. Similarly, 3 of 10 potential VP11/12 peptides, VP11/1266-74, VP11/12220-228, and VP11/12702-710, were selected as being highly recognized by CD8+ T cells from 10 HSV-1 seropositive ASYMP individuals, while CD8+ T cells from ASYMP individuals significantly reacted to 5 of 10 VP13/14 epitopes (VP13/14286-294, VP13/14504-512, and VP13/14544-552).

As an important initial phase in the development of an ocular herpes subunit vaccine, we next determined the capacity of these peptides to induce sustained CD8+ T-cell responses from “naturally” protected HSV-1-seropositive healthy ASYMP individuals (who have never had clinical herpes disease), but not from SYMP individuals, were selected from HSV-1 gB, VP11/12, and VP13/14 proteins.
responses in vivo. We tested the hypothesis that immunization with mixtures of ASYMPT synthetic peptides bearing these T-cell epitopes from gB, VP11/12, and VP13/14 would stimulate strong, functional, and multiepitope CD8⁺ T cells in the ocular herpes HLA-A*02:01 transgenic (HLA-Tg) rabbit model.

Selection of HLA-A*02:01 Tg Rabbits for Preclinical Evaluation of the Efficacy of HSV-1 ASYMPT CD8⁺ Peptide Vaccines Against Ocular Herpes

We first selected HLA-Tg rabbits with similar high expression of HLA-A*02:01 molecules, since expression of the rabbits’ own MHC class I molecules might interfere with the human HLA-A*02:01-restricted responses. All rabbits used had similar high expression of HLA-A*02:01 molecules in over 90% of PBMC by FACS (Figs. 1A, 1B). As expected, no HLA-A*02:01 expression was detected in wild-type (nontransgenic) rabbits (negative controls). The specificity of anti-human HLA-A*02:01 antibody was confirmed using an isotype IgG control. The high expression of HLA-A*02:01 molecules in the selected HLA-Tg rabbits should result in rabbit CD8⁺ T cells using the human HLA-A*02:01 molecules at the thymic selection and peripheral effector levels.

Frequent HSV-1 gB, VP11/12, or VP13/14 Epitope-Specific CD8⁺ T Cells Induced in HLA-Tg Rabbits by Mixtures of ASYMPT Peptides

To gain insight into the immunogenicity of HSV-1 asymptomatic peptide vaccines, and to obtain maximal information from limited numbers of animals, we analyzed the HSV-1 epitope-specific CD8⁺ T-cell responses in 3 groups of HLA-Tg rabbits (n = 10) each immunized with mixtures of 3 ASYMPT CD8⁺ T-cell peptides derived from gB, VP11/12, or VP13/14 molecule (i.e., gB 17–25, gB 342–350, and gB 561–569 in Group 1; VP11/12 66–74, VP11/12 220–228, and VP11/12 702–710 in Group 2; and VP13/14 286–294, VP13/14 504–512, and VP13/14 544–552 in Group 3, as shown in Table 1). Because concomitant induction of T helper CD4⁺ T cells is crucial in the priming and maintenance of HSV-specific CD8⁺ T cells, each mixture of CD8⁺ T-cell epitopes was delivered with PADRE, a promiscuous HSV-1 CD4⁺ T helper epitope that binds to most MHC class II molecules, including rabbit MHC class II molecules. As illustrated in Figure 2, 15 days after the second immunization each animal received an ocular challenge with HSV-1 (2 x 10⁵ plaque-forming units [pfu]/eye, strain McKrae).

The frequencies of CD8⁺ T cells were determined in PBMC from each animal, using human epitope-specific tetramers before immunization, 14 days after second immunization (i.e., /C₀ before HSV-1 challenge), and 9 and 21 days after challenge. As shown in Figures 3A and 3B and in Tables 2 and 3, one of the three gB peptides (gB 561–569), one of the three peptides from VP11/12 (VP11/12 220–228), and one of the three peptides from VP13/14 (VP13/14 504–512) elicited significantly higher frequencies of CD8⁺ T cells in the majority of immunized HLA-Tg rabbits compared to mock-immunized HLA-Tg rabbits (P < 0.01). The remaining six peptides from gB, VP11/12, and VP13/14 (i.e., gB 17–25, gB 342–350, VP11/12 66–74, VP11/12 702–710, VP13/14 286–294, and VP13/14 544–552) induced moderate to low frequencies of CD8⁺ T cells. The frequency of CD8⁺ T cells induced by each of the three immunodominant ASYMPT epitopes (i.e., gB 561–569, VP11/12 220–228, and VP13/14 504–512) was boosted.
following HSV-1 challenge (Fig. 3B), suggesting that CD8\(^+\) T cells induced by these synthetic “artificial” peptide epitopes were able to be recognized and be boosted by the “native” HSV-1 epitopes following HSV-1 challenge. Interestingly, most animals (6 of 10) developed CD8\(^+\) T cells against the VP11/12 220–228 peptide. Five of 10 animals showed significant responses to gB 561–569 and to VP 13/14 504–512 (Table 2). As expected, none of the control animals that received adjuvant alone (mock-vaccinated) showed significant increase in percentage of CD8\(^+\) T cells specific to any of the epitopes tested (Fig. 3B). Collectively, frequent CD8\(^+\) T cells were detected against each of the three HSV-1 Ags, pointing to induction of strong T-cell immunogenicity by mixtures of ASYMP epitopes.

### Induction of Polyfunctional CD8\(^+\) T Cells in HLA-Tg Rabbits by Mixtures of HSV-1 ASYMP Peptides

We next determined whether the mixtures of ASYMP epitopes from HSV-1 gB, VP11/12, and VP13/14 will induce functional CD8\(^+\) T cells in terms of IFN-γ, TNF-α-production, and CD107\(^+\) expression (i.e., cytotoxic activity). As shown in Figures 4A through 4C and in Table 2, 9 days after the challenge, at the time of acute infection, three of the nine peptides (one from each Ag) elicited significant IFN-γ-producing CD8\(^+\) T cells in PBMC from HLA-Tg rabbits. From the three peptides selected from VP11/12, VP11/12 220–228 elicited significantly higher IFN-γ-producing CD8\(^+\) T cells (Tables 2, 3). Lower, but significant levels of IFN-γ-producing CD8\(^+\) T cells were generated against VP11/12 702–710, but no cytotoxic activity was detected against the remaining VP11/12 246–254 peptide (Tables 2, 3). Similar results were obtained when CD8\(^+\) T cells were tested for production of TNF-α (Figs. 4D–F; Table 2). In addition, CD8\(^+\) T cells derived from immunized HLA-Tg rabbits showed cytolytic activity against gB 561–569 peptide and low but consistent cytotoxic T-cell responses against the remaining gB peptides (gB17–25 and gB 342–350; Figs. 4G-I; Tables 2, 3). As expected, none of the peptides elicited significant CD8\(^+\) T-cell responses in mock-vaccinated HLA-Tg rabbits (Fig. 4). Most animals (6 of 10) developed CD8\(^+\) T-cell responses against a single gB peptide (gB 561–569; Tables 2, 3). In contrast, 6 of 10 animals showed potent IFN-γ-producing CD107\(^+\)CD8\(^+\) cytotoxic T cells to two VP11/12 peptides (VP11/12 220–228 and VP11/12 246–254; Tables 2, 3). Interestingly, most animals developed CD8\(^+\) T-cell responses against all three VP13/14 peptides (Tables 2, 3). Six of 10 animals showed potent IFN-γ-producing CD107\(^+\)CD8\(^+\) cytotoxic T cells to VP13/14 504–512 while five showed significant response to VP13/14 544–552. Collectively, the results point to polyfunctionality of CD8\(^+\) T cells induced by mixtures of ASYMP epitopes from HSV-1 gB, VP11/12, and VP13/14.

### Protective Efficacy Against Ocular HSV-1 Infection and Disease

Groups of HLA-Tg rabbits (n = 10 mice per group) were immunized subcutaneously twice as above. As illustrated in Figure 2, two weeks after the final immunization, animals from all groups received an ocular HSV-1 challenge (2 \(\times\) 10\(^5\) pfu, McKrae strain) without scarification. The rabbits then were

---

**Figure 2.** Illustration of peptide immunization regimen, HSV-1 challenge, T-cell immunogenicity, and protective efficacy studies in HLA-Tg rabbits. Three groups of age-matched HLA-A*02:01 rabbits (n = 10 each) were immunized subcutaneously twice (on days 0 and 15) with CD8\(^+\) peptide epitopes as outlined in Materials and Methods. As a negative control, a fourth group of HLA transgenic rabbits (n = 10) received adjuvant alone (mock-vaccinated). Two weeks after final immunization, HLA-Tg rabbits were ocularly challenged (both eyes, without scarification) with 2 \(\times\) 10\(^5\) pfu of HSV-1 (strain McKrae) delivered as eye drops in 20 \(\mu\)L of tissue culture media. Rabbits were examined for ocular disease immediately before ocular challenge, and on days 1, 3, 5, 7, 10, 14, and 21 thereafter. Survival was determined in a window of 30 days after challenge. Tears from both eyes were collected by swabbing rabbit eyes with a Dacron swab on days 3, 5, and 7 after challenge for titration of viral load. Frequency and function of HSV-1 epitope-specific CD8\(^+\) T cells were analyzed on days –1, 9, and 21 post challenge.
assessed for up to 30 days post challenge for ocular herpes pathology, ocular viral titers, and survival.

As shown in Figures 5A and 5B, on day 10 after infection, the pathology clinical scores observed in the immunized groups were significantly lower compared to those recorded in the mock-immunized group ($P < 0.01$ for all). Furthermore, significantly less virus was detected on day 7 post infection (the peak of viral replication) in the eye swabs of the vaccinated groups compared to the mock-immunized group ($P < 0.01$, Fig. 5C). Most animals in the vaccinated groups survived infection (80%–70%) compared to only 30% survival in the mock-immunized group ($P < 0.05$; Fig. 5D). The mixtures of gB and VP11/12 peptides showed a trend to provide better protection than the VP13/14 peptide mixture.

Altogether, these results indicated that immunization with mixtures of ASYMP peptide CD8$^+$ T-cell epitopes from HSV-1 gB, VP11/12, or VP13/14 decreased ocular herpes disease, decreased virus replication, and protected against lethal ocular herpes in the HLA-Tg rabbit model of ocular herpes; and support HLA-A*02:01 Tg rabbits as a useful animal model for investigating the underlying mechanisms by which CD8$^+$ T cells specific to human HSV-1 CD8$^+$ T-cell epitopes mediate control of ocular herpes infection and disease.

**Frequent and Polyfunctional CD8$^+$ T Cells Specific to HSV-1 ASYMP Epitopes Are Associated With Reduced Corneal Herpetic Disease in Immunized HLA-Tg Rabbits**

Antibody depletion of CD8$^+$ T cells often is used in mice to determine if a protective immunity is CD8$^+$ T-cell dependent. Unfortunately, at this time, in vivo CD8$^+$ T-cell depletion studies are not feasible in rabbits because of the lack of a suitable in vivo depleting antibody. Thus, we determined...
whether the observed protection against recurrent ocular herpes was associated with frequency and function of CD8\(^+\) T cells specific to ASYMP HSV-1 gB, VP11/12, and VP13/14 epitopes.

The function of CD8\(^+\) T cells specific to HSV-1 gB, VP11/12, and VP13/14 epitopes was detected using specific tetramers together with intracellular staining for TNF-\(\gamma\) and IFN-\(\gamma\) and surface expression of CD107\(\alpha\)/\(\beta\), as described in Materials and Methods. There were significantly more HSV-specific IFN-\(\gamma\)- and TNF-\(\gamma\)-producing CD8\(^+\) T cells (Fig. 6, Table 3) in protected HLA-Tg rabbits compared to unprotected rabbits. Approximately two-thirds (73%, 69%, and 63%) of HSV-1 gB, VP11/12, and VP13/14 epitopes-specific CD8\(^+\) T cells in protected HLA-Tg rabbits, respectively, expressed at least two functions (i.e., production of IFN-\(\gamma\) and cytotoxic activity measured by expression of CD107\(\alpha\)/\(\beta\)), as described in Materials and Methods.

Altogether, these results suggested that TNF-\(\gamma\) and IFN-\(\gamma\)-producing HSV-specific CD107\(\alpha\)/CD8\(^+\) cytotoxic T cells might have a role in limiting ocular herpes infection and disease.

### DISCUSSION

Herpes simplex virus-1 ASYMP epitope-specific CD8\(^+\) T-cell responses assessed in vitro, correlate with less frequent and less severe ocular herpetic disease in HSV-seropositive individuals.\(^{4,16,25,26,28–33}\) We wanted to determine whether in vivo immunization with these human ASYMP CD8\(^+\) T-cell epitopes induces frequent and polyfunctional HSV-specific CD8\(^+\) T cells that reduce herpetic corneal disease. Using the new “humanized” HLA-Tg rabbit model of ocular herpes infection and disease, we characterized the T-cell immunogenicity and protective efficacy of nine human ASYMP CD8\(^+\) T-cell epitopes that we recently identified from three major HSV-1 Ags, namely, gB, tegument proteins VP11/12, and VP13/14.\(^{25,26}\) We showed that immunization with mixtures of HSV-1 ASYMP peptides elicited polyfunctional CD8\(^+\) T-cell responses in HLA-Tg rabbits and protected HLA-Tg rabbits from ocular herpes infection and disease following an ocular challenge. These preclinical results indicated that these ASYMP T-cell epitopes from glycoprotein gB, and from tegument proteins VP11/12 and VP13/14 are logical candidates to be included in the next generation of ocular herpes vaccines.

Although full-length proteins generally are regarded as ineffectivem epitogens for CD8\(^+\) T-cell responses,\(^{34}\) the 9-mer peptides used here have the optimal length for binding with high affinity to HLA class I molecules. There are several

### Table 3.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>No. Animals Immunized</th>
<th>Mean IFN-(\gamma)-Positive CD8(^+) T Cells</th>
<th>Mean TNF-(\gamma)-Positive CD8(^+) T Cells</th>
<th>Mean CD107(\alpha)/(\beta)-Positive CD8(^+) T Cells</th>
<th>Mean Tetramer Specific CD8(^+) T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB(^{1–25})</td>
<td>10</td>
<td>4.0 (1.6–9.3)</td>
<td>4.8 (2.8–7.2)</td>
<td>1.6 (0.7–3.5)</td>
<td>2.6 (0.8–6.5)</td>
</tr>
<tr>
<td>gB(^{342–350})</td>
<td>10</td>
<td>10.8 (3.2–16)(^a)</td>
<td>10.0 (3.2–16.9)(^a)</td>
<td>3.6 (1.3–5.2)(^a)</td>
<td>8.9 (3.9–12.1)(^a)</td>
</tr>
<tr>
<td>VP11/12(^{66–74})</td>
<td>10</td>
<td>4.0 (2.0–6.2)</td>
<td>4.5 (3.1–6.9)</td>
<td>1.3 (0.5–3.0)</td>
<td>2.8 (0.8–5.8)</td>
</tr>
<tr>
<td>VP11/12(^{220–228})</td>
<td>10</td>
<td>10.2 (2.1–14.7)</td>
<td>10.5 (2.8–15.2)(^\ast)</td>
<td>2.9 (0.9–4.6)(^\ast)</td>
<td>8.5 (4.9–13.2)(^\ast)</td>
</tr>
<tr>
<td>VP13/14(^{286–294})</td>
<td>10</td>
<td>3.8 (2.1–6.2)</td>
<td>4.2 (2.9–5.9)</td>
<td>0.8 (0.3–1.6)</td>
<td>2.0 (0.6–5.3)</td>
</tr>
<tr>
<td>VP13/14(^{404–512})</td>
<td>10</td>
<td>8.0 (2.3–12.5)(^\ast)</td>
<td>9.3 (3.6–14.3)(^\ast)</td>
<td>2.8 (1.6–3.9)</td>
<td>7.2 (3.6–10.2)(^\ast)</td>
</tr>
<tr>
<td>VP13/14(^{444–552})</td>
<td>5</td>
<td>4.3 (1.5–6.5)</td>
<td>3.8 (2.1–6.0)</td>
<td>1.5 (0.7–3.7)</td>
<td>2.6 (0.4–5.1)</td>
</tr>
</tbody>
</table>

\(^{\ast}\) Immunodominant epitopes.
advantages of a vaccine comprised of multiple peptide epitopes: HSV-1 ASYM epitopes can be included and HSV-1 SYM epitopes can be avoided, and epitopes from multiple viral proteins can be included. This is likely to produce even more efficacious protection than any one of the 3 epitope mixtures (each from a different viral protein) used here. Multiple HLA class I haplotypes, beside HLA-A*02:01, can be targeted (reviewed previously). Moreover, the mixtures of asymptomatic peptides vaccine did boost the number and function of local HSV-specific CD8+ T cells in protected HLA-Tg rabbits, while the number and function of local HSV-specific CD8+ T cells were significantly lower in unprotected HLA-Tg rabbits. These findings are in agreement with our recent report showing lower frequencies of functional HSV-1 gD epitopes-specific CD8+ T cells in protected HLA-Tg rabbits compared to more dysfunctional exhausted PD1+CD8+ and TIM-3+CD8+ T cells in unprotected HLA-Tg rabbits. Thus, the present results highlight the potential of mixtures of peptides vaccine for activating potent functional CD8+ T-cell responses against ocular herpes, compared to immunization with a single immunodominant peptide.

In these studies, mixtures of peptides, instead of individual peptides, were used because only small cohorts of HLA-Tg rabbits were available. A possible concern of a multipeptide...
vaccine is the possibility of epitope competition among the mixtures of epitope peptides for specific HLA-A*02:01 molecules. This could impair induction of a full spectrum of T-cell responses against all of the desired ASYMP epitopes. One approach to avoid this possibility would be to administer individual peptides at different injection sites. It is noteworthy that in this report, using peptide epitope mixtures for immunization, CD8\(^+\) T-cell responses were induced in HLA-Tg rabbits by 6 of the 9 peptides studied (Tables 2, 3). The polyfunctionality of induced CD8\(^+\) T cells may have a critical role in protection against ocular herpes infection and disease, since the quality of CD8\(^+\) T-cell responses, rather than their quantity (i.e., frequency of CD8\(^+\) T cells and magnitude of CD8\(^+\) T-cell responses) correlated better with protection.

To the best of our knowledge, the immunogenicity and protective efficacy of asymptomatic epitopes from glycoproteins B and D, and from tegument proteins, such as VP11/12 and VP13/14, have never been described in rabbits. Thus, the results of the present study revealed for the first time the immunogenicity and protective efficacy of several new CD8\(^+\) T-cell epitopes from many HSV-1 proteins, expanding the panel and diversity of HSV-1 Ags epitopes, and confirming that tegument proteins can induce strong CD8\(^+\) T-cell responses.

Traditional vaccine formulations using recombinant proteins are generally ineffective at induction of CD8\(^+\) T-cell responses.\(^3\)\(^4\) This limitation results from the basic biology of Ag processing and presentation of CD8\(^+\) T-cell epitopes, which necessitates endogenous synthesis and presentation in the context of HLA class I molecules. Through identification of protective human ASYMP CD8\(^+\) T-cell epitopes from HSV-1 proteins, herpes vaccine formulations can be simplified, enabling molecular-level vaccine characterization and improved safety profiles. We demonstrated the immunogenicity and protective efficacy of mixtures of several HSV-1 epitopes derived from 3 different HSV-1 proteins against ocular herpes infection and disease. This epitope-based approach, however, comes at the expense of decreased immunogenicity, requiring the addition of immunoadjuvants, as well as mixing and delivering of multiple epitopes from one or several Ags.\(^3\)\(^4\) Moreover, we found that HSV-1 ASYMP epitopes-specific polyfunctional CD8\(^+\) T cells induced by these molecular vaccines correlated with reduced corneal disease.

**Figure 4.** Continued.
It is estimated that approximately 450,000 adults in the United States have a history of recurrent herpetic ocular disease (symptomatic SYMP individuals), with approximately 20,000 individuals per year experiencing recurrent, painful, and potentially blinding ocular herpetic lesions.1,2,4–6 The seropositive SYMP and ASYMP individuals are different with regards to HSV-1 epitopes-specificity (the magnitude and the nature of their CD8⁺ T cells).¹–⁶,¹⁶,²⁴,²⁵,³⁹ Thus, a vaccine that converts the presumably nonprotective profile of CD8⁺ T cells seen in SYMP patients into the protective profile seen in ASYMP individuals will likely lead to a decrease in ocular herpes infection and disease. In the present study, we demonstrated that immunization with mixtures of peptide vaccines exclusively bearing human ASYMP gB, VP11/12, or VP13/14 epitopes, that are mainly recognized by CD8⁺ T cells from HSV-1 seropositive healthy ASYMP individuals who have never had clinical herpes disease,⁵–⁷ reduced infectious virus in tears and lessened ocular herpes following ocular challenge in prophylactically immunized HLA-Tg rabbits. This peptide vaccine excludes SYMP epitopes that are recognized mostly by CD8⁺ T cells from SYMP individuals with a history of numerous episodes of recurrent ocular herpes disease. It remains to be determined whether this ASYMP epitopes vaccine given therapeutically to latently infected HLA-Tg rabbits will significantly decrease virus reactivation from TG (virus shedding in tears) and/or recurrent ocular disease, and increase the numbers and functions of local HSV-1 gB, VP11/12, and VP13/14 epitopes specific CD8⁺ T cells over the existing immune response induced by the primary infection. Preclinical studies in HLA-Tg rabbits assessing the protective efficacy of these ASYMP epitopes vaccines in a therapeutic setting will be the subject of future reports.

Mice have been the animal model of choice for most immunologists, and results from mice have yielded tremendous insights into the role of T cells in protection against primary herpes infection.⁵,¹⁶,⁴⁰–⁴⁴ In addition, HLA-Tg mice can develop T-cell responses to human epitopes.⁴ From a practical standpoint, the size of rabbit cornea is significantly larger than those of mice and offer plentiful amount of tissues for phenotypic and functional characterization of HSV-specific T cells using individual tissues.¹⁷,⁴⁵–⁴⁸ In addition, compared to...
mice, the surfaces of the rabbit and human eye are relatively immunologically isolated from systemic immune responses. To overcome the hurdle that rabbits do not mount T-cell responses specific to human HLA-restricted human epitopes, we recently introduced a novel ‘humanized’ HLA-Tg rabbit model of ocular herpes in which the rabbits express HLA (HLA class I molecules). This novel HLA-Tg rabbit mounts CD8+ T-cell responses specific to HLA-restricted epitopes similar to humans. Since expression of the rabbits’ own MHC class I molecules might interfere with the human HLA-A*0201-restricted responses, and to ensure that all rabbits have high level of expression of HLA-A*0201 molecules in over 90% of their CD8+ T-cells, only those HLA-Tg rabbits with these characteristics were used in these studies. We previously reported that all gD epitopes that are recognized by CD8+ T cells from HSV-1 infected HLA-Tg rabbits are recognized by CD8+ T cells from HLA-A*0201-positive HSV seropositive humans. Thus, the HLA-Tg rabbit model will be useful for preclinical testing of candidate vaccines bearing human T-cell epitopes.

The human population is genetically diverse in terms of HLA haplotypes. Thus, a question of practical importance is the translation of the current immunological findings in a single HLA-Tg rabbit strain for the development of an epitope-based vaccine for a genetically heterogeneous human population. We chose the HLA-A*0201 haplotype in this study because: (1) >51% of humans, regardless of age, sex, ethnicity, and race, are this haplotype, and (2) we have HLA-A*0201 Tg rabbits.

**Figure 5.** Protective immunity against corneal disease, ocular herpes replication, and survival induced by SYMP gB, VP11/12, and VP13/14 CD8+ T-cell epitopes in HLA transgenic rabbits. Two weeks after the final immunization, all animals were challenged ocularly with 2 × 10^6 pfu of HSV-1 (strain McKrae) and examined for signs of ocular disease, virus titer, and survival in a window of 30 days after infection. (A) On day 10 after infection, the corneal epithelial cells of HLA-Tg rabbits were stained with topical dye (fluorescein or rose Bengal) to characterize ocular surface diseases and quantify their severity. Fluorescein and rose Bengal are instilled as a drop at a concentration of 0.1% to 1% at the surface of the eye. Rabbit eye images were taken under the correct color light using slit-lamp (blue for fluorescein and red for Rose Bengal). After staining the eyes were rinsed with PBS. The images show uptake of dye in the mock group and absence of the staining in the immunized group. (B) Clinical assessments by slit-lamp were made before inoculation, and on days 1, 3, 5, 7, 10, 14, and 21 thereafter. The examination was performed by investigators blinded to the treatment regimen of the rabbit eyes and scored according to a standard 0 to 4 scale: 0, no disease; 1, 25%; 2, 50%; 3, 75%; 4, 100%. (C) Virus titrations were determined from eye swabs on days 3, 5, and 7 post infection. Rabbits were swabbed with moist, type I-calcium alginate swabs and titrated on RS cell, as described in Material and Methods. The maximum virus titers obtained on day 7 are shown. (D) Survival was determined in a window of 30 days post challenge, as described in Material and Methods, using Kaplan Meier Test to show statistical significance between mock and immunized rabbits. Solid circles represent immunized and open circles represent mock-immunized rabbits. The results are representative of 2 independent experiments.
rabbits available for in vivo studies. Previous comparisons of HLA frequencies among cohorts of genital herpes patients showed associations of HLA-B27 and -Cw2 with symptomatic disease. In contrast, we have not detected any correlations between any particular HLA class I or class II haplotype and resistance or susceptibility to ocular herpes disease (unpublished data). Thus, we do not think the difference in ocular herpetic disease is due to a particular HLA haplotype. Although the high degree of HLA polymorphism often is pointed to as a major hindrance to the use of epitope-based vaccines, this can be overcome by the inclusion of multiple supertype-restricted epitopes, recognized in the context of diverse related HLA alleles, and by designing mixtures of peptide-based vaccines with higher epitope densities. Thus, broad population coverage can be established, providing that epitopes corresponding to multiple HLA supertype families are incorporated into the vaccine. A combination of nine HLA supertypes can provide greater than 99% coverage of the entire repertoire of HLA molecules. A multiepitope-based herpes vaccine could also include several T-cell epitopes present not only in one herpesvirus glycoprotein, such as gD, but also in several different proteins chosen to represent the HLA supertypes known to provide recognition in a large proportion of the global population. Such a multiepitope T cell–based vaccine would be broadly effective in the vast majority of individuals regardless of their HLA haplotype.

Whether the vaccine results obtained from the HLA-Tg rabbit model will correlate with vaccine results in humans remains to be determined in future studies. The advances in the present study offer great promise for mixture peptide-based vaccines. It will take time to optimize the parameters that influence immunogenicity of peptide-based herpes simplex vaccine, including route of immunization, method of antigen attachment, particle size, and composition (i.e., synthetic, or self-assembling, e.g., virus-like particles). A general principle is that immunogenicity is greatly enhanced by delivering peptide epitopes and TLR (or other innate immune receptor) activating substances in the same particle. Finally, it also will be important to determine the potency of mixture peptide-based vaccines in nonhuman primates and humans.
In conclusion, there are three principal findings in the present report. First, a human herpes vaccine that exclusively contains a mixture of human ASYMP CD8\(^+\) T-cell epitopes derived from HSV-1 gB, VP11/12, and VP13/14 provides protection in HLA-A*02:01 Tg rabbits against ocular herpes infection and disease. Second, frequent polyfunctional HSV-1 ASYMP epitope-specific CD8\(^+\) T cells were induced by mixtures of ASYMP human epitopes in protected HLA-Tg rabbits, suggesting their contribution in protection. Third, the study validates the HLA-A*02:01 Tg rabbit model of ocular herpes for preclinical testing of future herpes vaccine candidates bearing human ASYMP CD8\(^+\) T-cell epitopes against ocular herpes. Overall, this preclinical therapeutic vaccine study in HLA-A*02:01 Tg rabbits provides optimism for the evaluation of a T-cell vaccination approach using multiple minimal ASYMP epitope peptides and paves the way for the clinical testing of ASYMP CD8\(^+\) T-cell epitope-based vaccines against recurrent ocular herpes.

FIGURE 5. Continued.
Acknowledgments

The authors thank Alison Deckhut Augustine and Dale Long from the NIH Tetramer Facility (Emory University, Atlanta, GA, USA) for providing the Tetramers used in this study. The authors alone are responsible for the content and writing of the paper.

Supported by Public Health Service Research Grants EY019896, EY14900, and EY024618 from the NIH, The Discovery Center for Eye Research, and Research to Prevent Blindness.

Disclosure: R. Srivastava, None; A.A. Khan, None; J. Huang, None; A.B. Nesburn, None; S.L. Wechsler, None; L. BenMohamed, None.

References


Asymptomatic Ocular Herpes Vaccine

40. Wuest T, Farber J, Luster A, Carr DJ. CD4+ T cell migration into the cornea is reduced in CXCL9 deficient but not CXCL10 deficient mice following herpes simplex virus type 1 infection. *Cell Immunol.* 2006;243:85–89.


